Drug resistance in eukaryotic microorganisms

Drug Resistance in Eukaryotic Microorganisms

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Eukaryotic microbial pathogens are major contributors to illness and death globally but much of their impact can be controlled by drug therapy. However, as with prokaryotic microbes, the emergence of drug resistance has threatened these treatment efforts. Here, we discuss the challenges posed by eukaryotic microbial pathogens and how these are similar to, or differ from, the challenges of prokaryotic antibiotic resistance. The therapies used for several major eukaryotic microbes are then detailed and the mechanisms that they have evolved to overcome these described. The rapid emergence of resistance and the restricted pipeline of new drug therapies pose significant risks to global health and are particularly acute in the developing world. Nonetheless, we detail how an integration of new technology, biological understanding, epidemiology and evolutionary analysis can help sustain existing therapies, anticipate the emergence of resistance or optimise the deployment of new therapies.

The identification and use of antibiotics presents one of the great medical achievements of the 20th Century, saving countless lives by controlling the risk of infection from contagion, after injury, surgery or in immunosuppressed individuals. However, in only 80 years since the introduction of penicillin, resistance to a broad range of antibiotic drugs has become widespread, with the compounded risk from multi-drug resistant bacterial infections severely limiting treatment options. This has created justified concern and global attention, not only in the medical community but also at Government level, in the media and the public[1].

Whilst predominantly applied to control prokaryotic microbial infections, the threat of disease from eukaryotic microbes has also been contained by therapeutic drugs - preventing or controlling disease caused by eukaryotic parasites and fungi in both a human and animal health setting. These represent some of the most important disease-causing agents (Table 1), particularly in the tropics where the distribution of the pathogen is frequently linked to the distribution of the arthropods that act as disease vectors. Such vector-borne parasites include malaria (*Plasmodium* spp.) and kinetoplastid parasites (*Trypanosoma cruzi*, causing Chagas' disease; *Trypanosoma brucei gambiense* and *T. b. rhodesiense* causing human African trypanosomiasis (HAT), and 17 *Leishmania* spp. causing a variety of cutaneous and visceral diseases). Other clinically important protozoan parasite species not considered in this review are transmitted either orally (*Toxoplasma*, *Giardia* and *Entamoeba*) or venereally (*Trichomonas*). Distinct from the many obligate eukaryotic unicellular parasites, opportunistic fungal pathogens are global in distribution and include *Candida*, *Aspergillus* spp., *Cryptococcus* and *Pneumocystis spp*.

The control of these eukaryotic pathogens has often involved therapies predating the use of penicillin and in some cases with unacceptable toxicity profiles[2]. Nonetheless, as with the rise of antimicrobial resistance in bacteria, resistance has or is emerging in the therapies targeting these eukaryotic microbes, with potentially devastating consequences for exposed populations. This, however, has received far less attention despite some commonality in its underlying causes. In this perspective, we detail how the control of eukaryotic microbes poses both similar and distinct challenges to that of bacterial pathogens, the drugs used to combat these pathogens and the resistance mechanisms they are evolving. Finally we discuss how the latest methodological approaches can anticipate the emergence of drug resistance and support the development of new therapeutic approaches, either through the development of new drugs, the maintenance of existing therapies or through the use of alternative approaches to limit the spread of drug resistance.

Common challenges for the control of prokaryotic and eukaryotic microbial pathogens.

The challenges in the control of eukaryotic microbial pathogens share many similarities with bacterial infections. Both replicate more rapidly than their hosts, such that resistance can be selected within a relatively short timescale within a treated host population. This is exacerbated by inappropriate treatment profiles, leading to subcurative exposure in the context of infection[3]. Problems of sub optimal dosing are particularly acute when applied to tropical parasites. For example, for antimalarials, up to 35% of drugs may be of poor quality, have poor packaging and labelling or be falsified[4]. With lower than optimal concentrations of the active agent, this rapidly selects resistance in exposed populations, as does underdosing resulting from self-prescription. Where zoonoses are concerned, such as with African trypanosomes, parasite selection in livestock populations treated with trypanocides in a context where there is poor supply chain management, fraudulent provision or cost barriers to optimal dosing, can also lead to resistance emergence. This represents a significant threat where up to 50 million doses of trypanocides are used in sub-Saharan livestock annually, mainly as a preventative, and trypanocides represent 45% of animal health costs. Agricultural use of fungicides might also contribute to the selection of azole resistant *Aspergillus fumigatus*[5], mirroring the situation with antibiotic exposure in veterinary contexts for bacterial infections, where environmental contamination generates significant regulatory concern[6].

A further similarity between bacterial and eukaryotic microbial pathogens is the phenomenon of persister populations[7]. This is the survival of a fraction of the population of pathogens following exposure to a chemotherapeutic agent (or vaccine). These can then re-establish patent infection whilst remaining drug sensitive (see review[8]). The state of persistence is not heritable and resistance is not due to genetic alterations directly linked to rendering a drug ineffective. Rather, persistence is a physiologically active state involving pathogen response to the assault which is initiated upon demand. Persistence ensures incidental survival but does not future-proof a pathogen as genetically heritable resistance would. However, the combination of persisters and sub-optimal drug dosage might form an enhanced reservoir for the emergence of resistance and may even provide a population pre-disposed to evolve resistance more readily. An example of this relating to parasite dormancy is the resistance of *Plasmodium falciparum* to artemisinin (and other antimalarials such as mefloquine, atovaquone), which was first characterised by degrees of persistence followed by the emergence of genomic changes now causally associated with resistance (see below). Similarly, fungal infections (e.g. *C. albicans*) associated with biofilms are a good example of persister populations analogous to those in bacterial communities[9-11]. The duration of persistence can range from days (*P. falciparum*) to lifelong (e.g. *C. albicans).* Mechanisms of persistence vary – they may emerge spontaneously possibly through stochastic changes in gene expression that prepare a population of pathogens for survival in varying environmental conditions ("bet hedging"). This is best described in bacteria[12] but is a phenomenon recently characterised in *P. falciparum*[13]. Furthermore, environmental signals may induce persistence such as the nutrient starvation typically encountered by *C. albicans* in biofilms[9,14].

Distinct challenges for the control of prokaryotic and eukaryotic microbial pathogens.

Although bacterial and eukaryotic microbes share common features with respect to their responses to drug exposure, there are also differences that particularly challenge the control of eukaryotic pathogens. First, eukaryotic microbes are more similar to their hosts than prokaryotic pathogens in terms of their biochemistry and metabolism, genetic composition, cell architecture and biology. Consequently, drugs targeting eukaryotic microbes must focus on differences from the eukaryotic norm, or particular specialisms of each pathogen group. This restricts the cross-specificity of drugs, such that there are distinctions in sensitivity between different apicomplexans (malaria,

toxoplasma) or between the evolutionarily divergent trypanosomes, *T. brucei* spp. and *T. cruzi*. Comprising a different evolutionary kingdom, fungi have many differences from other eukaryotic microbial pathogens, again necessitating drugs to be developed for, and targeted to, a particular pathogen. This increases the challenges for drug development and inevitably constricts the new drug pipeline.

Second, many eukaryotic microbial pathogens have evolved a parasitic life style distinct from the opportunistic infections characteristic of most bacterial pathogens (but also fungi). The evolution of parasitism is often accompanied by the development of sophisticated immune evasion mechanisms, which promotes the impact of persister phenotypes described earlier. Specifically, bacteriostatic drugs can operate to clear infection in concert with the immune system[15]. However, drugs that generate cytostatic rather than cytocidal responses in infection with an immunosuppressive parasite can lead to recrudescence upon the removal of drug exposure. This, in turn, can predispose the population to the selection for drug resistance. Similarly the adaptation to an intracellular life style or particular body niche can protect parasites from drug exposure, a feature shared with some bacterial pathogens that have evolved to survive in cells rather than systemically (*Legionella*, *Mycobacteria*).

A third challenge relates to the clinical diagnosis and the screening for drug resistance in eukaryotic microbial pathogens[16]. In bacterial infections, screening for the sensitivity to antibiotics is straightforward and routine. In contrast, eukaryotic parasites can require highly-specialised growth media and considerable growth periods to determine their susceptibility or otherwise to potential drug therapies. Also, unlike bacterial susceptibility testing where a Minimum Inhibitory Concentration (MIC) is determined, most parasitologists report EC_{50}-values without providing the Hill slope of the growth inhibition curve or calculating the EC_{90} value. It is perfectly possible to obtain a resistant line with an identical EC_{50} to the susceptible isolate, yet that is still resistant due to a shallower Hill slope. As a consequence clinical diagnosis and the selection of the appropriate clinical management can be slow, or practically impossible in the context of all but the most specialised laboratories.

A fourth distinction from common bacterial infections is the economic challenge of treating diseases of the developing world. Diseases such as malaria, trypanosomiasis, leishmaniasis and cryptococcosis are common in the poorest parts of the world where the economic capacity to develop or deliver treatments are very limited and restricted to philanthropic and charitable donations, or the concerted actions of multi-Government agencies. This makes the threat of drug resistance even more acute, because there is not the financial incentive to develop new drugs to

replace those to which resistance emerges. Nonetheless, certain major pharmaceutical companies are increasingly engaged in Public Private Partnerships providing access to chemical compound collections and other resources to discover and develop new drugs for neglected tropical diseases. Excellent examples of this collaborative spirit include the Medicines for Malaria Venture (http://www.mmv.org/), the Drugs for Neglected Diseases initiative (http://www.dndi.org/) and the Tres Cantos Open Lab Foundation (http://www.openlabfoundation.org/).

One route to limit the impact of drug resistance has been the exploitation of combination therapies for parasitic infections. This approach has proved useful for cancer therapy as well as for the treatment of TB, leprosy and viral infections such as HIV. It has also been encouraged for parasitic infections, for example through artemisinin combination therapy[17,18] to limit the emergence and spread of artemisinin-resistant malaria, and for trypanosomes where nifurtimox/eflornithine combination therapy[19] is proving more robust than eflornithine-based therapy alone. However, combination therapies for parasitic diseases require the availability of more than one effective drug or drug class, which is not always the case. Moreover, combination therapies have been often embraced only when resistance is already detected to one of the front line monotherapies, allowing multidrug resistant parasites to be selected. Here, the use of drug combinations with different pharmacokinetics in plasma, as with artemisinin and piperaquine, can limit resistance emergence[20]. However, the cost of drugs for many parasites of the developing world can generate geographical discrepancy in the use of mono and combination therapies. Here, the efficacy of combination therapies can be threatened by ingression of resistant parasites selected under monotherapy.

The final challenge for eukaryotic microbes that differs from many prokaryotic and viral pathogens has been the failure to formulate and use effective vaccines to prevent infection[21]. Malaria research has focused intensively on vaccine development without transformative success, whereas for African trypanosomes the immune evasion mechanism employed by the parasite (antigenic variation) effectively renders vaccine approaches impossible. Other kinetoplastids have also proved challenging to produce safe effective vaccines, despite the widespread early use of ‘leishmanization’ for the cutaneous form of leishmaniasis, which has the risk of virulence in some individuals and immunosuppression[22]. Fungal pathogens have their greatest impact in immunocompromised individuals rendering vaccines potentially less useful. At present there are no licenced fungal vaccines; nonetheless, there are promising developments for adhesion-like substance 3 (Als3) and secreted aspartic protease 2

(Sap2) based vaccines, although concerns have been raised over their univalency and the potential for *C. albicans* to circumvent their efficacy[23].

Drugs used against different eukaryotic microbes and examples of the resistance mechanisms against them

Throughout evolution microorganisms have evolved numerous strategies to counteract cellular toxicity induced by diverse chemical stresses (xenobiotics, metals, reactive oxygen and reactive nitrogen species, etc). Many of these generic defences have been co-opted for drug resistance. Figure 1 summarises the major therapeutic agents used to target malaria, kinetoplastid parasites and fungi, highlighting the dates of introduction and the appearance of resistance for each. The principal methods of resistance (Figure 2) involve either reduction of the free drug level at the target site of action, alterations in the drug target reducing its drug binding affinity or over-expression of the target restoring its essential function. In the case of inhibition of a metabolic pathway, the essential end-product can be produced either by induction of an alternative pathway or by upregulation of a salvage pathway in order to obtain an essential metabolite from the host. Downstream consequences of target inhibition include damage to DNA, proteins and lipids such that upregulation of repair pathways can also contribute to resistance. Unlike bacteria, acquisition of resistance genes by lateral gene transfer on plasmids has not been observed for protozoan parasites or fungal pathogens. In Table 2 we summarise the drugs used to treat eukaryotic microbial pathogens, their mode of action and mechanisms of resistance where known. Below, we highlight specific examples where drug resistance or the threat of resistance challenges current control efforts.

Malaria:

The most successful antimalarial in history to date has been chloroquine (CQ), a 4-aminoquinoline derivative of quinine (itself the world's first mass-distributed antimalarial) and first synthesized in 1934[24]. CQ was cheap and remained effective for decades. However, due to massive overuse and suboptimal compliance, resistance to chloroquine emerged in Southeast Asia in 1957 and in South America in 1960, and-by the mid 1980's- it was barely possible to use even in Africa[25]. Whilst disputed by some[26] the leading candidate for resistance to CQ (CQR) is PfCRT (P. falciparum CQR transporter)[27]. However, despite reports that PfCRT functions as a chloride channel, a proton pump, an activator of Na^+/H^+ exchangers or a cation channel, the physiological function of PfCRT remains unclear[28]. Nonetheless, PfCRT is central to much

antimalarial resistance, the precise profile of which is modulated by associated mutations in other genes.

Artemisinin and its derivatives are fast acting but short-lived antimalarials that have been globally successful. In particular artemisinin-based combination therapies (ACTs, e.g. artemether-lumefantrine, artesunate-amodiaquine, and dihydroartemisinin-piperaquine) were recommended by the WHO in 2001 to ensure high cure rates of falciparum malaria and to reduce the spread of drug resistance to other front line drugs. However, clinical resistance was confirmed in 2008[29] characterised by a failure to rapidly clear parasites in patients around the Thai-Cambodian border[30,31]. Resistant parasites were characterized by transcriptomics[32], large scale whole genome sequencing (WGS) of clinical isolates[33,34] and classical generation of resistant mutants by in vitro culture followed by WGS[35]. This pinpointed multiple independent mutations in a gene encoding a Kelch propeller protein (Kelch 13) which was then causally linked to resistance by reverse genetics[36,37]. Large-scale genomic epidemiological evidence suggests that artemisinin resistance is not as straightforward as the simple acquisition of mutations in kelch13. Indeed, nonsynonymous mutations in ferredoxin, apicoplast ribosomal protein S10, multidrug resistance protein 2 and the chloroquine resistance transporter (PfCRT) also showed strong associations with artemisinin resistance[29]. These mutations appear to act as markers of a genetic landscape upon which artemisinin resistance-conferring kelch13 mutations are more likely to occur. These landscape mutations also correlate with the current geographical limits of artemisinin resistance[29]. This concept is further supported by additional genomic epidemiological evidence that demonstrates many of the 20 or so mutations in kelch13 that have been implicated in the SE Asian manifestation of artemisinin resistance are also found in African PF isolates. However, these mutations are present at no greater frequency in the African strains than other PF genes indicating a lack of selective pressure in that continent and that these strains lack the enabling genetic background observed in SE Asia[38].

Kelch propeller domain proteins are subcellular organisers of multiprotein complexes and indeed artemisinin resistance associated mutation of Kelch 13 results in its enhanced association with phosphatydylinositol-3-Kinase (PI3K)[39]. Experimental overexpression of PI3K results in enhanced artemisinin resistance and PI3P levels are predictive of resistance to artemisinin[39]. In addition, upregulation of the chaperonin complexes, PROSC and TRiC, involved in the unfolded protein stress response in other eukaryotes, may contribute to artemisinin resistance[32]. Worryingly, resistance to some of the various ACT regimens (involving lumefantrine and amodiaquine and PfCRT) is becoming evident[40-43]. However the framework for the rapid evaluation of genome evolution in the face of drugs is in place and will hopefully swiftly indicate any further potential mechanisms.

Human African trypanosomiasis:

The vast majority of reported cases of HAT are caused by *T. b. gambiense,* with less than 2% caused by *T. b. rhodesiense*[44]*.* Treatment involves either pentamidine or suramin for stage 1 infection (before CNS involvement) whereas melarsoprol, eflornithine or nifurtimox/eflornithine combination therapy are used once the parasite crosses the blood-brain barrier[2], the latter combination therapy reducing the duration of treatment regimens. Given the limited chemotherapeutic options for the treatment of HAT (Table 2), drug resistance could seriously compromise efforts to eliminate this epidemic disease as a public health problem[44]. Fortunately, resistance emergence for pentamidine has not been significant, despite continuous use of pentamidine since the 1940s, including a mass chemoprophylactic campaign in the 1950s in the then Belgian Congo. However, cross resistance to pentamidine and melarsoprol, used for stage 2 of infection, is frequently observed. Melarsoprol is a trivalent melaminophenyl arsenical which has a propensity to react covalently with vicinal dithiols, including the parasite-specific dithiol, trypanothione[45], to form a cyclic complex known as MelT[46]. Melarsoprol has a high incidence of severe (lethal) toxicities and high rates of treatment failures have been reported in the Democratic Republic of Congo, Uganda, Angola and Sudan[2]. Although therapeutic failure does not necessarily equate with drug resistance, it appears that the high relapse rate in northwest Uganda is associated with reduced susceptibility to melarsoprol[47,48]. The recent report that the aquaglyceroporin AQP2 appears to function as a transporter for large drugs such as pentamidine and melarsoprol was surprising given that Aquaglyceroporins are channels facilitating the passive transport of water and small neutral molecules across cell membranes. Nonetheless, there is strong evidence that AQP2 is indeed synonymous with the high affinity pentamidine transporter (HAPT1)[49], with a recent report indicating that pentamidine binds and inhibits the transporter and is then internalised via endocytosis[50].

Chagas' disease:

For *Trypanosoma cruzi,* an intracellular parasite with a wide tissue tropism, infection has three phases: an acute phase associated with high parasitaemia; an asymptomatic (indeterminate) phase lasting anywhere between 10-30 years, where parasitaemia is controlled by the immune response; and a chronic phase in about 30-40% of patients characterised by either cardiac disease or digestive disease (mega-oesophagus and mega-colon). For treatment, benznidazole and nifurtimox have significant activity in the acute phase[51] and benznidazole also eliminates parasitaemia in the indeterminate and chronic phases of the disease[52,53]. However, a large multi-centre, randomized trial of benznidazole for chronic Chagas' cardiomyopathy failed to significantly reduce

cardiac clinical deterioration through 5 years follow-up[53]. Whether this is due to differences in drug susceptibility, pharmacokinetic/pharmacodynamic issues or the pathophysiology of the disease is not known. The results of two recent clinical trials with azole ergosterol inhibitors, posaconazole and E1224 (a pro-drug of ravuconazole) have been equally disappointing[52,54,55].

Visceral leishmaniasis:

Treatment of visceral leishmaniasis (VL), cutaneous and mucocutaneous leishmaniasis is limited to four main drugs: pentavalent antimonial complexes (sodium stibogluconate and meglumine antimonate); amphotericin B (as deoxycholate or liposomal formulations); the aminoglycoside paromomycin; and the alkylphosphocholine miltefosine[56,57]. Treatment varies according to geographical location, the immune status and other co-morbidities of the patient, and the disease classification[58].

Of these treatments, widespread resistance to antimonial drugs is specific to Southern Asia and not in Sub-Saharan Africa or Brazil. Indeed, antimonial drugs are not recommended in India or Nepal due to treatment failures commencing in the 1990s and now reported to be as high as 60% in some regions[59]. This has been attributed to inappropriate treatment in an unregulated private health system or to the use substandard antimonial drugs. However, Southern Asia is the only region where arsenic exposure and widespread antimonial resistance co-exist. Thus, environmental pollution and exposure of patients to arsenic in food and drinking water was proposed as an alternative hypothesis[60]. Arsenic and antimony are both metalloids and selection of leishmania parasites for resistance to trivalent arsenic results in cross-resistance to trivalent antimony in vitro[61], but its physiological relevance was uncertain. Chronic exposure of infected mice to arsenic in drinking water at environmentally relevant levels demonstrated that it is possible to generate resistance to pentavalent antimony *in vivo*[62]. A retrospective clinico-epidemiological study identified a trend towards increased treatment failure in arsenic exposed patients, but failed to reach statistical significance[63].

Resistance to antimonials is multifactorial and most of the mechanisms shown in Figure 2 have been implicated in *Leishmania*. Studies on experimental and clinical resistant isolates strongly support the hypothesis that trypanothione plays a pivotal role in antimonial resistance. However, none of the following mechanisms are universal in resistant isolates. Decreased biological reduction of Sb^{V} to Sb^{III} has been reported in

resistant leishmania amastigotes[64] and two candidate "antimony reductases" identified, although genetic[65,66] and proteomic studies[67,68] have not identified any changes in either TDR1[69] or ArsC[70]. The mechanism of uptake of Sb^{V} is not known, but modulation of expression of aquaglyceroporin 1 (AQP1) affects Sb^{III} susceptibility[71-73]. AQP1 copy number and expression levels correlate with susceptibility to Sb^{III} in some, but not all, clinical isolates[74,75]. However, interpretation of this observation is complicated by the fact that AQP1 is located on chromosome 31, which is frequently trisomic or tetrasomic[76] in these mosaic aneuploid parasites[77]. Upregulation of trypanothione and ancillary biosynthetic pathways has also been observed in genomic[65,66] and metabolomic[78,79] studies. MRPA is responsible for ATP-dependent efflux of Sb^{III} as a thiol conjugate into membrane vesicles[80] and a homodimeric ABC half-transporter (ABCI4) is one possible candidate for efflux across the plasma membrane[81].

Miltefosine, the only oral treatment for VL, was first approved for use in India in 2002. However, a decade on there is an increasing rate of clinical relapse[82,83], which threatens to undermine the Kala-Azar Elimination Program in the Indian subcontinent. Stable resistance is readily generated in the laboratory with no cross-resistance to other anti-leishmanial drugs[84,85].

Fungi:

Several classes of antifungals are used clinically (Table 1, Table 2) – each with very different drug resistance profiles. The oldest antifungals are the polyene macrolide antibiotics, exemplified by amphotericin B, which remains a front-line choice of a broad spectrum agent for fungal infections of unknown aetiology. Amphotericin deoxycholate has significant nephrotoxicity which is significantly ameliorated in lipid carrier formulations such as AmBisome, which also has potent anti-*Leishmania* activity. As with other eukaryotic pathogens, resistance to antifungal drugs has become an increasing important clinical problem[86,87]. A few recognised cases exist of inherent resistance of specific fungi to specific antifungals, but mostly resistance is due to induced changes and mutations.

The imidazoles and more modern triazoles (collectively known as the "azoles") constitute the main class of antifungals used in the treatment of infections. Various modifications of the triazole ring have generated a series of antifungals including fluconazole (used mainly in the treatment of *Candida* infections), and itraconazole, voriconazole, posaconazole, ravuconazole and the recently licenced isavuconazole which have improved activity against *Aspergillus* and filamentous fungal species. These compounds have important differences in antifungal potencies, spectrum of

activities, bioavailability, drug interactions and toxic potential. For example, some patients treated with voriconazole suffer from photosensitivity and an elevated risk of skin carcinoma[88]. Other sterol inhibitors include the allylamines squalene epoxidase inhibitors and phenylmorpholine Erg24 D14 reductase and Erg2 D8-D7 isomerase inhibitors that are used topically against dermatophytic infections for which clinical resistance is low.

Although some fungi such as *Candida krusei* are inherently azole resistant, multiply triazole resistant strains are now emerging[89,90] as well as strains with cross resistance to azoles and echinocandins suggesting worrisome multi-drug resistance (MDR) phenotypes in medically important fungi[91]. A threat from multi-azole resistant strains of *A. fumigatus* may have arisen under the selective pressure of agricultural azole fungicides and subsequent transmission of azole resistant strains to the clinic by spore dispersal[92-95]. The prevalence of these alleles is increasing in Europe and now in other parts of the world[90,96,97]. In *Candida* mutants harbouring azole resistance have a fitness deficit[98]; however, MDR strains of *Aspergillus* do not seem to have significantly decreased fitness implying they may become stably represented in the environment.

The most recently developed major class of antifungal are the echinocandin antibiotics of which caspofungin, micafungin and anidulafungin are used clinically. These have similar pharmacokinetic properties although a new echinocandin (CD101-formerly Biofungin) is in clinical trials and has improved stability *in vivo* and requires less frequent i.v. dosing. Echinocandins are fungicidal against *Candida* species and fungistatic or fungicidal against *Aspergillus* causing hyphal or bud tip lysis but they are not efficacious against *Pneumocystis jiroveci* and some other species.

Hsp90-mediated changes in drug tolerance have also been implicated in determining echinocandin sensitivity[99]. Recently, multi-drug azole/ echinocandin resistance has been identified in fungi and this is particularly frequent in strains of *C. glabrata* which is common in patients with haematological malignancies and solid tumours[100,101]. These MDR strains of *C. glabrata* become reliant on i.v. amphotericin treatment, and since this agent has poor penetration into urine such infections are essentially untreatable.

Outstanding challenges and future prospects

This review began by highlighting the similarities and differences between drug resistance emergence in prokaryotic and eukaryotic microbes. The control of the

emergence of drug resistance for eukaryotic microbial pathogens also has similarities and distinctions from prokaryotic drug resistance, and our challenge for the future is to ensure best practice is employed for both groups. One effective mechanism to control drug resistance spread in bacterial pathogens is the application of appropriate antibiotic stewardship, applying the right drug at the right dose, at the right time, for the right duration. This approach operates effectively where there is well-regulated healthcare, effective and rapid screening, a selection of available drugs as contingency and the necessary education and engagement between the patient and healthcare provider. Moreover, bacterial drug resistance is a global phenomenon where resistance selected through poor stewardship in one geographical area may be contained by stringent practices in other areas, or combatted by an investment in new pharmaceutical development in wealthy countries. These containment measures are inevitably less effective where primary care is limited or too expensive, education is lacking or where the diseases involved do not have direct impact in the developed world. In consequence, the limitation of many eukaryotic pathogens to the poorer parts of the world makes a co-ordinated response to resistance emergence more difficult to achieve.

The drivers of resistance emergence are also more difficult to mitigate for many eukaryotic pathogens. As highlighted earlier, drug provenance and effective delivery is a significant challenge in the developing world. The latter is a particular challenge for prospective mass drug administration programmes where delivery to a population on a broad or local scale, if incomplete, can counteract its intention to contain the spread of existing resistance in target regions. A further complication in low and middle-income countries is the effects of co-infection or malnutrition in populations treated with drugs targeting a particular pathogen (discussed in [102]). Notably, the pharmacokinetic behaviour of drugs in malnourished individuals may be variable and unpredictable leading to inadvertent under-dosing, driving resistance emergence. When combined with immunosuppression induced by many parasites, or the hospital-induced immunosuppression of patients that become susceptible to fungal infection, drug concentrations that would clear infections in the context of a robust immune system may fall short in its absence. The ecological balance between distinct pathogens in patients with coinfections can also lead to unanticipated consequences, where the removal of one pathogen can create a niche exploited by a distinct pathogen or where the normal interactions between pathogens with each other, and with the immune system, is perturbed with drug pressure. The resistance mechanisms selected in drug treated populations can also alter pathogen phenotypes with the risk of enhanced virulence.

Although the factors that drive drug resistance are well known, it remains essential to identify when drug resistance arises and to respond rapidly and effectively. As with health care, surveillance is a key challenge for diseases in the developing world, where populations may be inaccessible, reluctant to engage or where treatment failure can have multiple causes beyond the emergence of drug resistance. Moreover, resistance can show considerable variation amongst populations or in different geographical settings. Here, accurate and rapid detection is critical to understand resistance epidemiology and thereby the best treatment to deliver, but this can be difficult to achieve. Despite this, developments in field PCR assays and next generation sequencing permit the sensitive identification and tracking of emergent resistance, allowing earlier control responses than could be previously achieved. Hence, an integration of improved therapeutic delivery and treatment monitoring are critical control points to reduce resistance emergence, in tandem with the discovery of the relevant resistance mechanisms and the search for new drug therapies. These combined approaches span from the individual scientific researcher to clinician, to health agency, to government and population, which must be well-integrated, and alert, with effective and rapid communication between distinct levels to allow appropriate responses to be put into action if needed.

Fortunately, whilst drug resistance is emerging in many eukaryotic microbial pathogens, new tools and methodologies are being developed to (i) predict resistance mechanisms, (ii) to identify modes of drug action and potential escape pathways and (iii) to understand pathogen biochemistry as a means to discover new potential therapies. With respect to drug resistance, the advent of cost-effective and rapid genome resequencing allows signatures of selection to be identified[103-106], whilst genome-wide RNAi screens allow the mapping of resistance pathways[107,108], and overexpression libraries[109] can assist with drug target deconvolution through selective screens. These genetic tools are complemented by improvements in proteomics such that adaptations accompanying drug resistance can be pinpointed, providing information on resistance mechanisms, and potential diagnostic tools to detect resistance emergence[110]. Combined with the improved sensitivity and resolution of metabolomics analysis[111], biochemical pathways can also be mapped in the context of drug exposure, allowing bypass mechanisms to be highlighted, if present. These each provide the essential early warning systems necessary to identify and combat the spread of drug resistance. Furthermore, certain combination therapies might offer novel transmission blocking strategies: very recently resistance to the antimalarial atovaquone, a component (with proguanil) of the widely used and successful treatment marketed as Malarone, has been further characterised. Resistance mutations that

appear during the target blood stage infection localise to the mitochondrial protein cytochrome b, one of the few proteins encoded by the highly reduced *Plasmodium* mitochondrial genome. All atovaquone resistance mutations examined generate a deficient mitochondrion and a parasite that, whilst viable in the blood, is incapable of development in the mosquito and thereby cannot be transmitted[112]. Thus despite the fact that resistance to atovaquone might arise repeatedly, each incident is isolated. Drugs that target cytochrome b could form part of combination therapies that are self-limiting in terms of spread of drug resistance and may delay any transmission of resistance that arises to the drug it is partnered with.

Concluding remarks

Drug resistance in eukaryotic microbes is an increasing global problem that threatens the advances in healthcare over the last 50 years. This mirrors the situation for bacterial and viral pathogens but is particularly acute given the abundance of eukaryotic pathogens in the poorest regions of the world. These countries have the least capacity to respond to resistance emergence through the development of new drugs vaccines and diagnostics, whilst developed countries lack financial incentives to assist. Nonetheless, there are opportunities to respond to this threat due to the distinct biology of many major eukaryotic pathogens and the discoveries made in basic research focused on their biology. Furthermore, many eukaryotic microbes are arthropod-borne diseases, such that targeting transmission can be a route to pathogen control not available for opportunistic pathogens. This can take the form of transmission-blocking vaccines or drugs targeting *Plasmodium*[113] or the application of vector control measures such as insecticide impregnated bed nets[114], peri-domestic and indoor residual insecticide spraying[114,115], tsetse traps[116], or improved housing[117]. Sterile insect release is also a route to limiting the vector population and so restricting disease spread[118,119]. Eukaryotic microbes have also, like some bacterial pathogens, been found to show co-operative and social behaviours to optimise their establishment and transmission in their hosts or vectors[120,121]. These social responses can control parasite density or the development of transmission stages[122-124], such that blocking or mimicking signals for communication or their transduction pathways provides new routes to limit the impact of the pathogens using strategies that might be less susceptible to resistance emergence.

Whether or not new targets or new approaches can be identified, there is a real need to optimise the delivery and deployment of drugs. Control of drug quality, distribution and supply of cost-effective drugs is crucial. Also the application of both

555 epidemiological modelling and evolutionary theory to guide drug treatment policies is
556 important in prolonging the life span of drugs and thereby maximising the return on the
557 considerable cost associated with developing and introducing a new drug. Targeted
558 therapy as opposed to mass drug administration is key to limiting the emergence of
559 resistance, or containing resistance when it is detected. This requires an integration of
560 epidemiology, diagnosis, detection and supply chain control as well as investment in a
561 pipeline of new therapeutics ready to be deployed when resistance inevitably emerges.
562 Only through slowing resistance emergence and accelerating new drug discovery will
563 the control successes achieved against eukaryotic microbial pathogens be sustained.
564

Table 1.

Diseases caused by eukaryotic microbes, their vectors and front-line treatment options. Several of the parasitic pathogens are arthropod-transmitted, and in these cases the responsible vector is shown. Fungal pathogens are predominantly opportunistic.

Table 2

Modes of action and mechanisms of drug resistance in eukaryotic microbes

Figure Legends

Figure 1 Timelines for emergence of drug resistance in parasitic diseases (A) and Fungi (B). The darker bar represents the time from first widespread clinical use to the first year drug resistance was suspected or confirmed. The shading indicates that certain drugs are still in use for particular indications or in specific geographical locations. Abbreviations: S-P, sulfadoxine-pyrimethamine; PPQ, piperaquine; ACTs, artemisinin combination therapies; NECT, nifurtimox eflornithine combination therapy; L-AMB, liposomal amphotericin B; MLT, miltefosine. For fungal pathogens, insensitive or resistant strains have been identified shortly after the introduction of all of the major classes of antifungal agents. In the case of amphotericin B, there remains very little resistance – and differences in sensitivity mainly reflect the relative inherent sensitivity of different species to this agent.

Figure 2 Molecular mechanisms of drug-resistance.
Eukaryotic microbial pathogens can exhibit drug resistance through reducing the overall intracellular concentration of the drug (less uptake, more efflux), by inactivating or failing to activate the drug, or by sequestering the drug away from its target. Resistance can also be mediated by reducing affinity of the drug for the target by mutation or by reducing the drug effect by overexpression of the target. Salvage and by-pass pathways can also lower the overall impact of the drug action, as can the activation of pathways in order to repair any damage caused.

598

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Table 1. Diseases caused by eukaryotic microbes, their vectors and front-line treatment options

Disease	Pathogen group	Vector	Pathogen	Front-line treatments [a]
Malaria	apicomplexan	Anopheline mosquitoes	*Plasmodium falciparum*	**Uncomplicated *P. falciparum* malaria**: Artemisinin combination therapies (ACTs) • Artemether + lumefantrine • Artesunate + amodiaquine • Artesunate + mefloquine • Dihydroartemisinin + piperaquine • Artesunate + sulfadoxine + pyrimethamine **Severe malaria:** Parenteral (or rectal, children < 6 years) artesunate followed by oral ACT (i.m. artemether or i.m. quinine if artesunate unavailable)
			P. vivax, P. ovale, P. malariae or *P. knowlesi*	**Blood stage infections:** Chloroquine (except in areas of chloroquine resistance) ACTs (except pregnant women and infants < 6 months) **Radical cure of liver (hypnozoite) infection:** Primaquine (close medical supervision with G6PD-deficient patients)
African trypanosomiasis	kinetoplastid	Tsetse flies	*Trypanosoma brucei gambiense* (chronic form)	**Haemolymphatic stage** (no CNS involvement): Pentamidine (i.m.) **CNS stage**: Nifurtimox (oral) / eflornithine (i.v.) combination therapy (NECT) (Melarsoprol if NECT unavailable)
			T. b. rhodesiense (acute form)	**Haemolymphatic stage** (no CNS involvement): Suramin (i.v.) **CNS stage**:

				Melarsoprol (i.v.)
American trypanosomiasis	kinetoplastid	Triatomine bugs	*Trypanosoma cruzi*	Benznidazole Nifurtimox
Leishmaniasis	kinetoplastid	Phlebotomine sandflies	**Visceral disease** *Leishmania donovani* *L. infantum* **Mucocutaneous disease** *L. braziliensis* *L. panamensis* **Cutaneous disease**, e.g. *L. major* *L. tropica* *L. mexicana* *L. amazonensis*	**Visceral disease:** Amphotericin B (as liposomal or deoxycholate complex, i.v.) Miltefosine (oral, contraindicated in pregnancy) Paromomycin (i.m.) Sodium stibogluconate (SSG) or meglumine antimonate, parenteral (except India and Nepal) SSG plus paromomycin (East Africa) **Mucocutaneous:** SSG (systemic) **Cutaneous:** SSG (intralesional) Paromomycin (ointment) Miltefosine
Invasive Candidiasis	fungal	opportunistic	*Candida albicans* *Candida glabrata* *Candida parapsilosis*	Echinocandins, Fluconazole, Liposomal Amphotericin B
Aspergillosis	fungal	opportunistic	*Aspergillus fumigatus*	Voriconazole (Amphotericin B formulations; caspofungin; micafungin; posaconazole; itraconazole)
Pneumocystis pneumonia	fungal	opportunistic	*Pneumocystis carinii*	Sulfamethoxazole-Trimethoprim (clindamycin-primaquine)
Cryptococcal meningitis	fungal	opportunistic	*Cryptococcus neoformans*	Amphotericin B plus flucytosine Amphotericin B plus fluconazole

[a] Second line treatment options are given in parentheses
Data from WHO [58,125-127] and other sources [57,128,129]

Table 2. Modes of action and mechanisms of drug resistance in eukaryotic microbes

Pathogen	Drug and date of resistance reported	*Drug class* and Mode of action	Resistance mechanism
Plasmodium	Chloroquine (CQ) 1957 (SE Asia) 1960 (S America) Mid 1980s (Africa)	*4-Aminoquinoline* Chloroquine interferes with the detoxification of haem into chemically inert haemozoin resulting in accumulation of toxic CQ ferric haem complex and subsequent parasite lysis[130].	K76T[27] mutation in a Digestive Vacuole-sited, ATP-dependent, 10 transmembrane domain transporter PfCRT (P. falciparum CQR transporter)[27]; a range of more than 30 different mutations might interact epistatically[131-133]. These stimulate the active efflux of CQ by mutant PfCRT or the passive efflux of diprotonated CQ[133]. Other genes contributing to resistance include: the P multidrug resistance transporter 1 (PfMDR1) homologue; multipass transmembrane transporter CG2; and PfNHE1 and a sodium hydrogen antiporter also associated with quinine resistance[134]. The specific genetic background of the parasite and the range of mutations in genes other than PfCRT are also key to the manifestation of CQR[135]. An independent mutation in PfCRT (C350R) can reverse CQR and also increase susceptibility of the parasite to other antimalarials (mefloquine, quinine and lumefantrine but not piperiquine[136]). The mutation N326D confers increased resistance to the antimalarial amodiaquine[40]
	Mefloquine 1982 (Thailand)	*Quinoline-4-methanol* Blockade of haemozoin formation and binding to phospholipids	PfMDR1 is associated with mefloquine resistance[137] but may also modulate CQR through compensatory mutations that counteract PfCRT mutations that compromise parasite fitness[138].
	Artesunate Dihydroartemisinin Artemether	*Sesquiterpene lactone endoperoxides.*	Dormancy resulting in an extended ring stage phase of development in the erythrocyte promotes resistance[30-32].

	2008[29] (SE Asia)	Form a carbon-centred free radical or reactive electrophilic intermediate that alkylates a number of malaria proteins[139] after activation by haem or free iron.	Multiple independent mutations in a gene encoding a Kelch propeller protein (Kelch 13) confer resistance[33-37]. This results in its enhanced association with phosphatydylinositol-3-Kinase (PI3K), which is subsequently under-ubiquitinated and accumulates along with its lipid product, phosphatidylinositol-3-phosphate (PI3P). The specific genetic background of the parasite and the range of mutations in genes other than *kelch13* may also be key to the manifestation of resistance to artemisinin[33]
	Sulfadoxine / Pyrimethamine 1967 (Thailand); 1980s (Africa)	*Antifols.* Sulfadoxine – inhibition of dihydropteroate synthase (DHPS) Pyrimethamine – inhibition of dihydrofolate reductase (DHFR) Synergistic effect on thymidylate synthesis	Decreased affinity of both drugs for their respective targets. Resistance to sulfadoxine involves DHPS point mutations., DHPS variant A437G confers moderate resistance, with the additional mutations S436F plus A613S conferring a high level resistance[140]. Pyrimethamine clinical resistance involves DHFR point mutations at S108N in Africa and SE Asia. Additional mutations that confer high level resistance are N51I and C59R[141] Increased GTP-cyclohydrolase (CNVs) enhances folate biosynthesis compensating for loss of fitness[141]
	Proguanil	DHFR inhibitor	High level resistance to cycloguanil (a metabolite of proguanil) involves DHFR mutation of serine 108 to threonine. The triple mutations (C59R, S108N and I164L) confer cross resistance to both pyrimethamine and cycloguanil[142].
	Atovaquone (in combination with proguanil for prophylaxis or treatment)	Cytochrome b inhibitor	Effective resistance to atovaquone involves one of a range of mutations in *cyt b* most commonly Y268S. Other mutations associated with such resistance include I258M, Y268C, M133I and V259L[143]
	Suramin	*Naphthylamine trisulfonic acid*	Laboratory-generated resistance mediated through the silencing of invariant surface glycoprotein (ISG75), the AP1 adaptin complex,

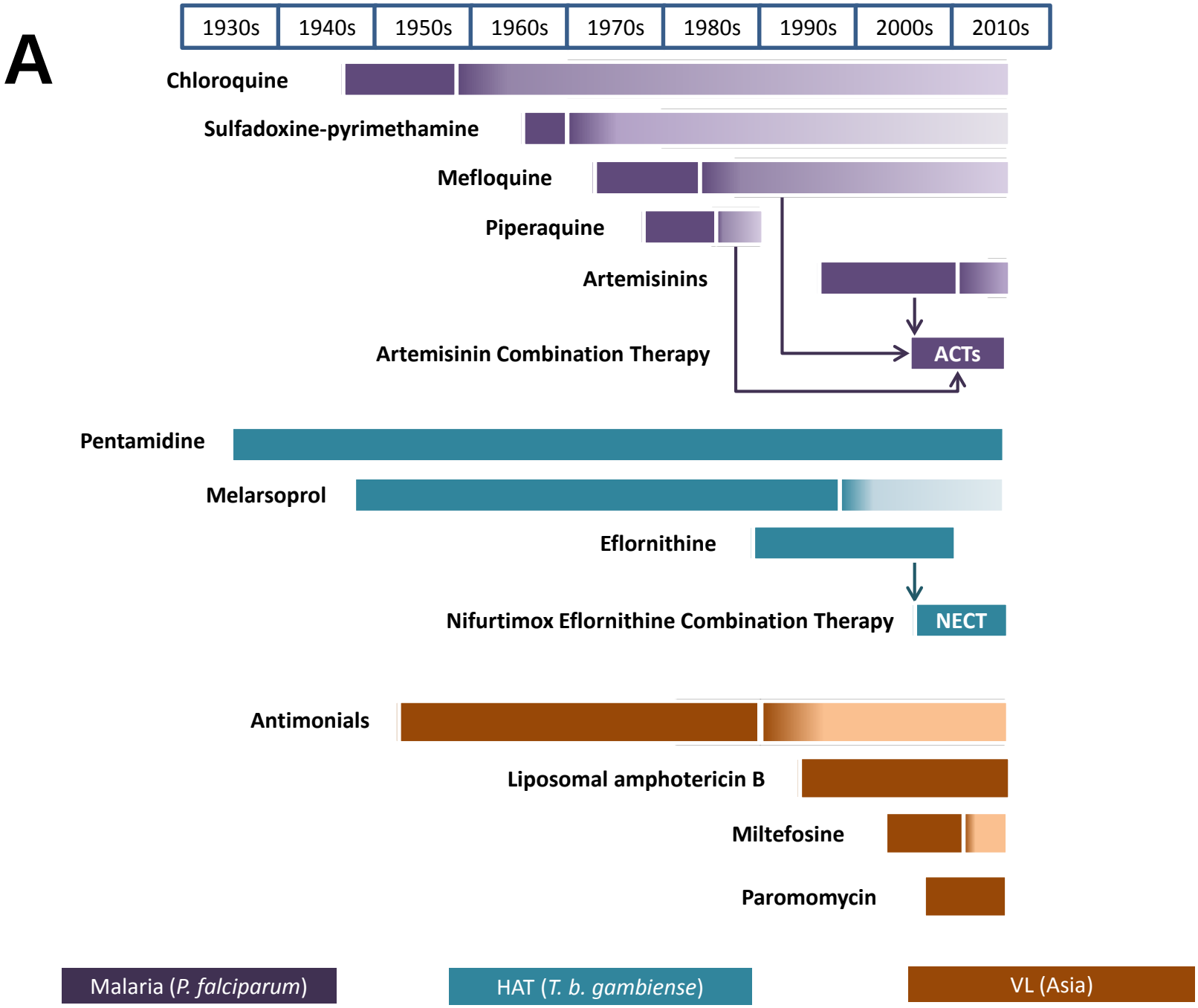
African Trypanosomes		Mode of action unknown	lysosomal proteases and major lysosomal transmembrane protein, as well as spermidine and N-acetylglucosamine biosynthesis[108].
	Pentamidine Clinical resistance is not significant.	*Diamidine* Mode of action unknown	Resistance is associated with loss of uptake on the P2 adenine/adenosine transporter[144], (AT1)[145]. Cross-resistance between melaminophenyl arsenicals and diamidines is mediated by aquaglyceroporin 2 (AQP2) [146]. A chimeric AQP2/AQP3 gene is associated with cross resistance to melarsoprol and pentamidine in laboratory-generated[49,146,147] and clinical isolates[148,149]
	Melarsoprol Treatment failures have been reported in the Democratic Republic of Congo, Uganda, Angola and Sudan[2]	*Trivalent melaminophenyl arsenical.* Forms a cyclic complex with trypanothione known as MelT[46]. Inhibits trypanothione reductase and no doubt other targets.	Resistance is associated with loss of uptake on the P2 adenine/adenosine transporter[144,145]. A non-functional mutant has been identified in melarsoprol-resistant field isolates[150]. See also AQP in pentamidine section.
	Eflornithine (difluoromethyl-ornithine)	*Fluorinated amino acid.* Mechanism-based inhibitor of ornithine decarboxylase, required for biosynthesis of polyamines and trypanothione.	Laboratory-generated resistance is due to loss of a non-essential amino acid transporter[151,152]. There is no detected resistance in *T. b. gambiense,* but there is inherent resistance in some clinical isolates of *T. b. rhodesiense*[2].
	Nifurtimox (poor efficacy as monotherapy; used in combination therapy with	*Nitrofuran* Prodrug activated by an oxygen-insensitive mitochondrial nitroreductase (NTR)[153] to	A genome-scale RNA interference screen identified NTR and a number of other genes possibly associated with NTR function[108]. NTR is also the key resistance determinant in laboratory-generated lines[156,157] showing cross resistance to fexinidazole an oral nitro-imidazole currently undergoing Phase II/III clinical trials for HAT.

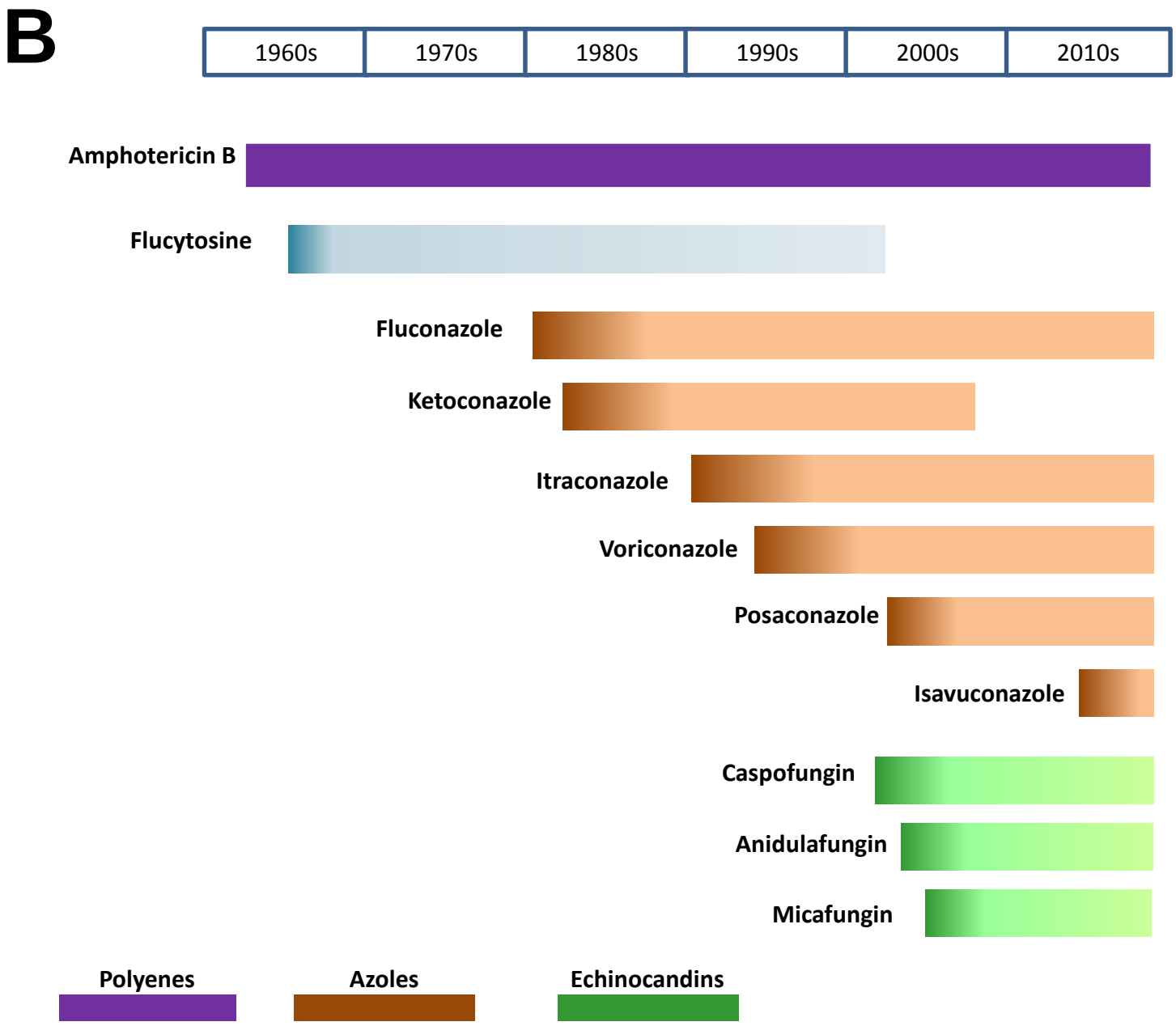
	eflornithine [NECT])	form highly reactive drug metabolites[154] that kill trypanosomes via unknown mechanisms[155].	
South American Trypanosomes	Benznidazole, Nifurtimox (natural resistance in some *T.cruzi* isolates)	*Nitroheterocyclics* Benznidazole is activated by mitochondrial NTR[153,158] to form electrophilic drug metabolites[159,160]	Drug efflux via an ABCG-like transporter[161] The NAD(P)H flavin oxidoreductase (old yellow enzyme) is downregulated in resistant lines[162,163]. However, this enzyme does not reduce benznidazole and only reduces nifurtimox under anaerobic conditions[164].
Visceral Leishmaniasis	Sodium stibogluconate, Meglumine antimonite 1990s widespread resistance in India and Nepal. Not widespread in Sub-Saharan Africa or Brazil	*Pentavalent antimonials* Sb^{V} is reduced to Sb^{III} to attack intracellular amastigotes. Likely to bind multiple targets including trypanothione reductase[165,166], tryparedoxin peroxidase[167] and CCHC Zinc finger proteins[166].	Selection for resistance to trivalent arsenic results in cross-resistance to trivalent antimony in vitro[61], and in vivo[62]. Resistance is multifactorial through several mechanisms: • Decreased reduction of Sb^{V} to Sb^{III} • Sb^{III} is taken up via an aquaglyceroporin[73] and modulation of expression of aquaglyceroporin 1 affects Sb^{III} susceptibility[71-73]. • Elevated Intracellular trypanothione levels[168] or increased biosynthetic potential[65,66,78,79] . • Increased levels of tryparedoxin peroxidase confer resistance to Sb^{III} [169] and are found in clinical resistant isolates[167] • MRPA (also known as PgpA or ABCC3), a member of the ATP-binding cassette (ABC) transporters, is amplified in some resistant lines[170-172] and sequesters Sb^{III} in an intracellular vacuolar compartment close to the flagellar pocket[80]. • chaperones and stress related proteins are upregulated[67,68] , potentially reducing or repairing cellular damage induced by antimonials[173]

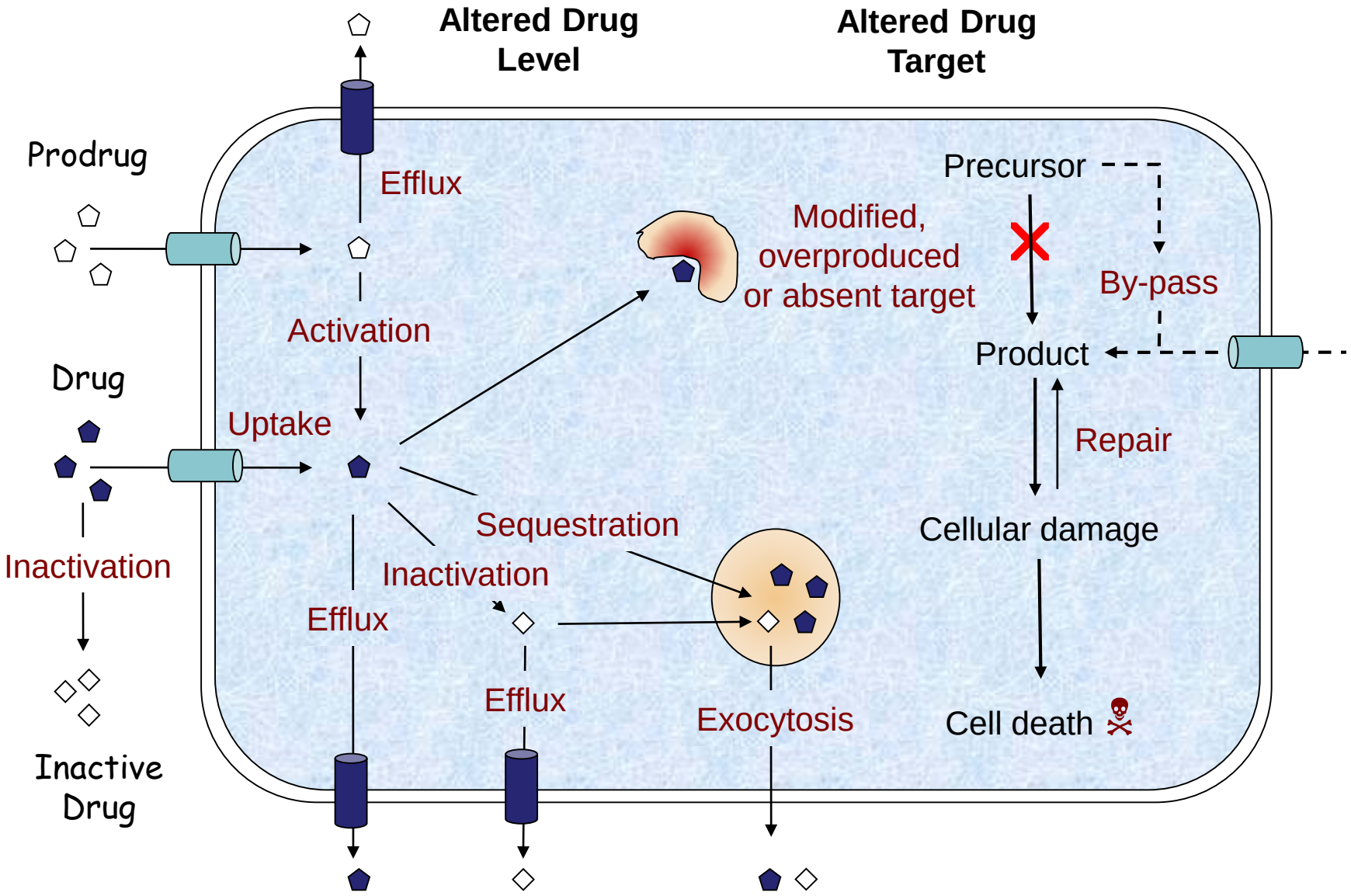
	Paromomycin	*Aminoglycoside* Inhibition of protein synthesis	Added to WHO essential medicines list in 2007. No significant clinical resistance. Laboratory-derived resistant lines show decreased drug uptake and increased expression of ribosomal proteins[174].
	Miltefosine 2012 (Indian subcontinent)	*Alkylphosphocholine* Miltefosine significantly perturbs lipid metabolism[175-177], but the targets and precise mechanism of action are not fully understood[178]	Resistance involves either: loss-of-function mutations or under-expression of an aminophospholipid translocase (LdMT)[179-181] or its regulatory subunit LdRos3[182]; or drug efflux by ABC transporters[183,184]. Laboratory-generated resistant lines show alterations in lipid metabolism and gene expression [85,185], but WGS in another study identified mutations only in the miltefosine transporter, pyridoxal kinase and an α-adaptin-like protein[176].
	Amphotericin B (deoxycholate or liposomal formulation)	*Polyene macrolide antibiotics* See below	No significant clinical resistance reported
Fungi	Amphotericin B, amphotericin deoxycholate	*Polyene macrolide antibiotics;* Binds ergosterol more avidly than human cholesterol disrupting the semipermeable membrane causing leakage of essential metabolites and the collapse of electrochemical gradients. Binding of low density lipoprotein receptors and amphotericin-mediated oxidative damage may also contribute.	Laboratory mutants with lower ergosterol content are less sensitive to amphotericin B, but are rare clinically. *Aspergillus terreus* is intrinsically less amphotericin sensitive but resistant strains have a normal ergosterol content suggesting that membrane permeability may not be the only mechanism of amphotericin action[186]. Binding to ergosterol might contribute to its mode of action[187].

	Fluconazole, Itraconazole, Voriconazole, Posaconazole, Ravuconazole, Isavuconazole	*Azoles*; Bind haem-groups and inhibit the P450–mediated 14α-demethylation (Erg11p or Cyp51p) of lanosterol in the ergosterol biosynthetic pathway. Leads to impaired membrane permeability, membrane protein action and cell wall synthesis[188].	Resistance involves the overexpression of drug efflux pumps and point mutations in the target *ERG11 / CYP51A* gene product, along with promoter mutations in these genes[189-191]. Changes in the levels of three main efflux pumps Cdr1, Cdr2 and Mdr1 and mutations in the genes encoding the Tac1, Upc2, Pdr1 and Mrr1 transcription factors required for efflux pump upregulation, represent major causes of decreased drug sensistivity[192,193]. This type of azole resistance can be acerbated by isochromosome formation and aneuploidy which can increase the copy number of key resistance genes such as *ERG11* and *TAC1*[194-196] . Interference with RNA polymerase II interacting Mediator-complex can re-sensitize Pdr1 dependent regulation of drug efflux pumps[197] Chaperone Hsp90 can mitigate against stress induced damage[198] and also contribute to multidrug resistance with Echinocandins. TR34/L98H and the more recently identified TR46/Y121F/T289A alleles that confer clinical azole resistance are likely to have arisen from environmentally generated mutations
	Caspofungin, Micafungin, Anidulafungin, Cd101 (formerly biofungin)	*Echinocandins*; Cyclic hexapeptides with an antifungal bioactive lipid side chain that binds the fungal specific β-1,3-glucan synthase Fks cell membrane proteins, disrupting cell wall integrity.	Resistance through point mutations in two major hotspots in the β-1,3 glucan synthase genes *FKS1* - and, in *C. glabrata*, *FKS2*[86,101,199], these reducing drug binding[57,181,182]. Upregulation of cell wall chitin can protect cell wall damage [184-186]. Hsp90 chaperone can mitigate against stress induced damage[170]
	Flucytosine (5-fluorocytosine)	*Fluoropyrimdines*; converted to 5-fluorouracil	Resistance results from mutations in the genes encoding cytosine permease transporter, cytosine deaminase, which converts 5-FC to 5-

		by cytosine deaminase which becomes incorporated into RNA resulting in inhibition of DNA synthesis.	fluorouracil or the uracil phosphoribosyl transferase required to convert 5-fluorocytosine into a substrate for nucleic acid synthesis[200]. Their impact is lessened by the use of 5FC in combination therapy.


A
1930s
1940s
1950s
1960s
1970s
1980s
1990s
2000s
2010s
Chloroquine
Sulfadoxine-pyrimethamine
Mefloquine
Piperaquine
Artemisinins
Artemisinin Combination Therapy
ACTs
Pentamidine
Melarsoprol
Eflornithine
Nifurtimox Eflornithine Combination Therapy
NECT
Antimonials
Liposomal amphotericin B
Miltefosine
Paromomycin
Malaria (P. falciparum)
HAT (T. b. gambiense)
VL (Asia)


B
1960s
1970s
1980s
1990s
2000s
2010s
Amphotericin B
Flucytosine
Fluconazole
Ketoconazole
Itraconazole
Voriconazole
Posaconazole
Isavuconazole
Caspofungin
Anidulafungin
Micafungin
Polyenes
Azoles
Echinocandins


Altered Drug Level
Altered Drug Target
Prodrug
Efflux
Precursor
Modified, overproduced or absent target
By-pass
Activation
Product
Drug
Uptake
Repair
Sequestration
Inactivation
Cellular damage
Inactivation
Efflux
Efflux
Exocytosis
Cell death
Inactive Drug